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A STUDY OF MESSENGER RNA IN REGENERATING RAT LIVER

JOSEPH A. DONADIO

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A Study of Messenger RNA in Regenerating Rat Liver

Joseph A. Donadio, A.B.

Johns Hopkins University, 1962

A Thesis Presented to

the Faculty of the School of Medicine

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INTRODUCTION

The concept of the functional relationship of DNA and RNA to protein synthesis is not a new one. Caspersson[1] proposed a general theory concerning this issue for the intact cell. He stressed that all protein synthesis required the presence of nucleic acids. Also, he stated that the most important nucleic acids were in the chromosomes and of the deoxyribose type. Finally, Caspersson pointed to the nucleus as the cellular center for the formation of proteins. Expanding on these premises, he proposed that the euchromatin, rich in DNA and of genetic importance, controlled the synthesis of specific, complex proteins representing the products of genes. Heterochromatin - especially that which was localized in the nucleolus - controlled the synthesis of histone-like proteins rich in basic amino acids. It was these substances which represented the major portion of the nucleolus. These basic proteins would then diffuse toward the nuclear membrane, overcome its barriers, and induce in the perinuclear cytoplasm an intense production of pentosenucleoproteins. These cytoplasmic pentosenucleotides then served to induce the synthesis of cytoplasmic proteins. Brachet[2] in writing about the biologic role of pentose nucleic acids concluded that there was good evidence that the nucleus played an important part in the PNA and nucleotide metabolism. However, he felt that it could not be stated with certitude whether or not nuclear PNA was a direct precursor of cytoplasmic PNA. It was his impression that indeed if such a transfer of nuclear PNA to the cytoplasm existed, it would involve a more complex phenomenon than that of simple diffusion. In addition, Brachet

felt that nuclear PNA was not the sole precursor of cytoplasmic PNA even though it had been shown by Brachet[3] and Linet and Brachet[4] that the nucleus was necessary for the prolonged maintenance of microsomes in enucleated organisms. Nevertheless, it was these early experiments which focused more and more on the implications of nucleic acids and microsomes on protein synthesis.

During this very same period, it was discovered that *in vitro* systems for protein synthesis could be constructed. Siekevitz[5] demonstrated the presence of high specific activity protein in the microsomal fraction when D L-alanine C^{14} was incubated with a rat liver homogenate. Significant incorporation did not occur when either microsomes or mitochondria were incubated with radioactive alanine. However, when mitochondral and microsomal fractions were incubated together in the presence of radioactive alanine, incorporation resulted. Zamecnik and Keller[6] were successful in the incorporation of several C^{14}-labeled amino acids into protein in a cell-free homogenate prepared from rat liver. The system consisted of a labile microsome-rich fraction, a stable, soluble, non-dialyzable fraction, the labeled amino acids, and an ATP-generating system. This incorporation system was found to be inhibited by ribonuclease and desoxycholate. Another cell-free protein synthesizing system was used by Schweet[7] which yielded evidence for the synthesis of a specific protein. C^{14}-labeled leucine, isoleucine, and valine were incorporated in ratios consistent with those in crude hemoglobin in a system containing microsomal particles from rabbit reticulocytes and soluble enzymes.

These results then provided evidence that the amino acid sequence of the final protein is determined in the microsomal particle. Thus the connection between RNA and protein synthesis was more firmly established. The localization of protein synthesis was relegated to the microsome - which was now known to be made up of ribonucleoprotein particles. The *in vitro* systems for protein synthesis indicated that one was dealing with a rather autonomous system. This thesis persisted for several years until questions were finally raised as to whether the RNA portion of the ribosome, which was found to be 50% RNA and 49% protein[8], could really transmit the DNA-derived genetic information or, more specifically, order the amino acids in prescribed sequence.

The base distributions of the nucleic acids were therefore studied[9]. Comparison of the analytical data on DNA and RNA composition in microorganisms demonstrated that the RNA composition did not vary as considerably as did the DNA in relation to the species. It followed then that the RNA composition displayed no marked conformity with that of the DNA. The appropriate question was quickly put forth. Can the view of a direct genetic connection between DNA and RNA in the cell be adhered to without reservation. An affirmative answer was difficult to assume in light of the marked variations in base composition of the DNA's and the rather uniform nature of the base composition of the RNA's. Nevertheless, it was not excluded that some small percentage of the total RNA was dependent upon DNA for its structure and, thus, would serve

as the genetic link between the highly species-specific DNA and the rather monotonously similar non-species-specific RNA.

As a reaction to the findings of the dissimilarity of the RNA to the DNA, claims appeared that in vitro protein synthesis showed dependence on DNA as well as RNA. Tissières[10] improved on the E. coli cell-free protein synthesizing system of Lamborg and Zamecnik[11] largely by regulating the Mg^{++} concentration, thereby stabilizing the ribosomal preparation. Using this system, the incorporation of C^{14} amino acids into peptide linkages was inhibited not only by ribonuclease and chloromycetin, but also by deoxyribonuclease. Thus, the importance of DNA in the cell-free protein synthesizing system was indicated.

During this same productive period, the enzymes catalyzing a DNA-primed RNA synthesis - consistent with the incrimination of DNA to RNA information transfer - were gradually elucidated. In 1955, Grunberg-Manago and Ochoa[12] reported the isolation of the enzyme polynucleotide phosphorylase which catalyzed the synthesis of highly polymerized ribonucleotides from 5' - ribonucleoside diphosphates with the release of inorganic phosphate. This enzyme by itself, however, did not provide a mechanism for the formation of RNA with a specific sequence of nucleotides. DNA-polymerase was first described in 1956 by Kornberg, Lehman and Simms[13]. This enzyme catalyzed a net synthesis of deoxyribonucleic acid which was dependent upon the presence of all four deoxynucleoside triphosphates, magnesium ions, and "primer" DNA. Weiss, stimulated by the

importance of specific ribonucleic acid molecules as carriers for individual amino acids in protein synthesis[14, 15] and the widely held view that RNA might provide templates for the synthesis of specific proteins, undertook an investigation of a new type of reaction for the incorporation of ribonucleotides. He demonstrated the enzymatic incorporation of ribonucleoside triphosphates into the interpolynucleotide linkages of RNA by an enzyme system isolated from rat liver nuclei[16]. Since this enzyme system required all four triphosphates, the reaction resembled, in this respect, the action of DNA-polymerase rather than that of polynucleotide phosphorylase. It is also interesting that preincubation of the enzyme with extremely small quantities of deoxyribonuclease completely inhibited the system, whereas similar treatment with ribonuclease was not as effective. Chamberlain and Berg, examining alternate mechanisms for RNA synthesis, particularly one in which DNA might dictate the nucleotide sequence of the RNA, reported the isolation of an RNA polymerase from E. coli which, in the presence of DNA and the four naturally occurring ribonucleoside triphosphates, produced RNA with a base composition complementary to that of the DNA[17]. Similar results emanated from the laboratories of Hurwitz et al.[18] and Stevens[19].

Further information on the participation of DNA in the synthesis of RNA was obtained by nearest-neighbor analysis of the RNA synthesized by a partially purified bacterial enzyme which required DNA - Micorcoccus lysodeikticus - by Weiss and Nakamoto[20, 21]. They showed that different DNA's - mammalian,

salmon, sea urchin, bacterial, and phage - altered the position of cytidylate in the newly assembled RNA chain and that the ribonucleotide had an average base composition similar to the "primer" DNA used. Then Hall and Spiegelman[22], capitalizing on the results of Marmur et al.[23] and Doty et al.[24], demonstrated specific complex formation between single-stranded T2-DNA and the RNA synthesized subsequent to infection of E. coli with bacteriophage T2. No such hybrid formation was observed with heterologous DNA even if it had the same overall base composition as the T2-DNA. It was concluded, therefore, that the T2-DNA and T2-specific RNA formed hybrids because they possessed complementary nucleotide sequences. Similar findings were reported by Geiduschek et al.[25] christening their DNA-dependent RNA, complementary RNA or C-RNA. This, they felt, was functionally similar to messenger-RNA (M-RNA) as defined by Jacob and Monod[26]. These observations, therefore, served to clarify the necessity for and the function of DNA in the process of in vitro protein synthesis since the supernatant fractions used in those experiments were shown to contain the DNA-dependent RNA. The concept of the continuous formation of newly synthesized template RNA was thus strongly considered.

Quite independently and significantly influential for the inevitable change in attitude toward the importance of the ribosome in the role of protein synthesis were a series of experiments that stemmed from an observation by Cohen in 1948[27]. He showed that cells infected with T2 bacteriophage did not appear to synthesize

or metabolize RNA, despite a rapid synthesis of DNA in the cells. Hershey et al.[28] in 1953 demonstrated that the DNA that accumulated in bacteria infected with T2 bacteriophage was of the distinctive viral type. Moreover, in a later paper, Hershey[29] isolated a metabolically active RNA fraction in the infected bacteria. The P^{32} used to identify this species of RNA was noted to be extremely rapidly incorporated into RNA, reaching a peak approximately five minutes after infection. Interestingly, the synthesis of this RNA quickly fell to a low level during the period of entry of the P^{32} into DNA. The author felt that the metabolic activity of this RNA clearly distinguished this material from the bulk of the RNA that was formed in the cell prior to infection. Little significance was attached to this finding and it was not until the series of observations of Volkin and Astrachan[30, 31] that the identification of the fast turnover RNA was established. They suggested that the bacteriophage infection induced the bacteria to synthesize this rapid turnover RNA. They elucidated the base composition of this RNA by obtaining the percentage of total RNA radioactivity associated with each of the mononucleotides. The proportions were found to be quite different from the chemically obtained base compositions of E. coli RNA. Furthermore, and most importantly, the analogous deoxyribonucleotides of T2 bacteriophage DNA had relative proportions similar to those described for the radioactive RNA of bacteriophage-infected bacteria[32]. A certain amount of time was needed for the implications of these sets of key experiments to become manifest.

Perhaps, much of the credit for the knowledge leading to the interpretation of the Volkin-Astrachan experiments[30, 31] must be delegated to Jacob and Monod[26]. In their paper, where they discussed the genetic regulatory mechanisms involved in protein synthesis, they postulated a hypothetical intermediate in protein synthesis which they called messenger RNA. The qualifications of such an RNA species according to Jacob and Monod[26] were the following:

1. The fraction would be a polynucleotide.
2. There would be heterogeneity with respect to molecular weight.
3. The base composition would mimic that of DNA.
4. There would be a temporal association with ribosomes.
5. A very high turn-over rate would be characteristic.

The rate of information transfer from the DNA to the ribosome, then, would depend upon the activity of the gene in synthesizing the messenger or upon the activity of the messenger in synthesizing the protein. Thus, the high turn-over RNA in phage infected cells with a base composition similar to phage DNA[31] must have served indeed as the "messenger" for the transfer of genetic information from phage DNA to the protein synthesizing centers. This assumption implied that the same protein-forming centers which in uninfected cells synthesized bacterial protein also functioned in infected cells to synthesize phage protein according to the new structural information provided by phage DNA via M-RNA.

This hypothesis was strongly supported by observations made by Brenner et al.[33]

with T4 infected E. coli. Uninfected cells were grown in the presence of N^{15}. They were then infected and resuspended in N^{14} medium. Following infection, they were exposed to short pulses of P^{32} or S^{35} and the ribosomes were analyzed in density gradients. The striking results were:

1. No dectectable amounts of ribosomal RNA were synthesized after infection.
2. Labelled M-RNA formed after infection became associated with unlabelled ribosomal particles formed before infection.
3. Newly formed, phage-determined protein was found to be associated with the 70 S particles before it appeared in the soluble protein fraction.

These findings strongly suggested that phage protein was synthesized by bacterial ribosomes formed before infection and associated with phage-determined M-RNA. The structural information for the phage protein could not have resided in the ribosomes. Therefore, the obvious alternative was that it was provided by the M-RNA. Thus, the concept of the ribosome serving as a mere carrier for the information-laden template RNA was established.

Pursuing still further the development of the thesis for messenger RNA in the coding process and the recession of the ribosome to the role of an inert carrier in protein synthesis, two more experiments will be cited. Gros et al.[34] demonstrated an RNA component in non-infected bacterial cells which was

physically distinct from the ribosomal RNA. This RNA behaved exactly as did the post-infection phage-specific RNA with respect to sedimentation constants and attachment to ribosomes in high magnesium ion concentrations. The base composition of the RNA was different from that of ribosomal RNA and approached that of DNA. This nicely demonstrated the normal sequence of events in the normal cell as was first elucidated in the phage infected cell. The classic experiments of Nirenberg and Matthaei[35] delineated clearly the status of the ribosome in protein synthesis. They used an E. coli cell-free protein synthesizing system wherein the ribosomes were pretreated with DNA-ase and subjected to a preliminary incubation. These depleted ribosomes in the presence of template RNA from E. coli, yeast, tobacco mosaic virus, and synthetic polynucleotides were shown to incorporate amino acids significantly better than before purification.

Thus the basic elements governing protein synthesis had been carefully dissected apart and identified by numerous ingenious experiments almost exclusively from microorganisms. The stage was now set for a more careful, detailed, and mechanistic characterization of that exciting entity, messenger RNA, in mammalian tissues.

The reticulocyte system in mammalian tissues was particularly examined and discussed. It was there that a stable template was indicated to exist[36]. However, as more experiments were accumulated and other mammalian systems examined, it was concluded that template stability in the mammalian cell was not the rule and

was perhaps even the exception. Relatively rapid RNA turnover was found by Cheng[37] in human amniotic cells grown in tissue culture. He exposed the cells to a five minute pulse of H^3-cytidine and submitted the phenol extracted RNA to a sucrose gradient zone centrifugation. The radioactivity peaks of the rapidly labeled RNA did not coincide precisely with the ultraviolet absorbancy peaks of the RNA. Also there was significant heterogeneity of the molecular weights of the rapidly labeled RNA. Georgiev[38] isolated a DNA-like RNA from the nucleo-chromosomal apparatus of mammalian cells. He demonstrated that if the nuclei were extracted with phenol at temperatures below 60 degrees, only RNA of the G-C type - identical to that of ribosomal RNA - was extracted. However, extraction at 60-65 degrees liberated another fraction of RNA of the A-U type. The RNA of the A-U type approximated the base composition of DNA. Also, using P^{32} incorporation into the nucleolo-chromosomal RNA, it was found that the relative specific activity for high temperature, nucleolo-chromosomal rat liver RNA was higher than that for the corresponding ribosomal RNA. Hiatt[39] pretreated normal and regenerating rat livers with C^{14} orotic acid and isolated RNA from both the nucleus and the cytoplasm. A heterogenous, rapidly labeled fraction, ranging in size from 6 to 30 S was found in the nuclei and not in the cytoplasm. The base composition of the rapidly labeled RNA in the nuclei differed from that of total nuclear RNA and also was dissimilar to the base composition of DNA. Its base composition was suggestive of enrichment with

respect to nucleolar RNA, however. On the basis of its size, the rate of labeling, and its distinctive base composition, this RNA fraction was interpreted to resemble the bacterial messenger RNA. The authors concluded further that its apparent absence from the cytoplasm indicated that a rapidly renewable messenger might exist in liver nuclei as contrasted to the existence of a relatively stable template in the cytoplasm. They cautioned nevertheless that before an RNA fraction could be incriminated as fulfilling the role of the biologic messenger, it must be shown capable of directing protein synthesis.

The isolation of a nuclear RNA fraction resembling DNA in its base composition was effected by Sibatani et al.[40] Their experiments concerned the isolation of thymus nuclear RNA, which they showed had a high rate of synthesis or turnover by allowing incorporation of C^{14}-orotic acid and C^{14}-adenine. Also, the base composition of this nuclear RNA resembled that of the DNA. By dint of its structure and its extreme metabolic activity, this RNA was considered to be highly suspect in transmitting the genetic information from DNA to the synthetic sites on the ribosomes. The authors felt that this RNA was the mammalian equivalent of the "phage-specific RNA" which was found to appear after bacteriophage infection of E. coli - the messenger RNA[26, 30, 33, 34]. Thus, a species of RNA having a high metabolic activity and a base composition reflecting that of DNA thereby resembling its counterpart in the bacterial protein-synthesizing systems was finally established for mammalian cells.

During the past three years, experiments have been designed to demonstrate further this RNA's capability of fulfilling the obligations for a messenger as proposed by Jacob and Monod[26]. Therefore, not only were the criteria of rapid rate of turnover and nucleotide composition similar to that of the DNA of the cell searched for and obtained, but also, and perhaps most critically, the ability of the RNA to stimulate the incorporation of amino acids into proteins by ribosomes was held to be the almost _sine qua none_ of the candidate RNA's achieving the status of messenger RNA.

Barondes in 1962[41], using the _E. coli_ cell-free protein synthesizing system of Nirenberg and Matthaei[35], demonstrated that rat liver nuclear RNA was far superior to microsomal RNA in stimulating amino acid incorporation into protein. Although the amount of protein synthesized was too small to characterize, the authors speculated that proteins resembling mammalian proteins were synthesized. Hoagland and Askonas[42] studied an RNA cytoplasmic fraction from the livers of normal and regenerating rats. This material stimulated amino acid incorporation in microsomes obtained from rat liver. This cytoplasmic RNA was labeled more rapidly _in vivo_ with P^{32} than were the other cytoplasmic RNA fractions, and it was found to sediment in a sucrose gradient more rapidly than soluble RNA and more slowly than did the ribosomes. Preparations of this same RNA from the regenerating animals proved to be more active than those from normal liver. Thus, this RNA possessed certain properties in common with bacterial messenger RNA.

However, this RNA differed from bacterial M-RNA in that it was insensitive to the action of ribonuclease.

Brawerman[43], using a phenol extraction procedure with alkaline buffers (pH 8.5 and 9.0), was able to demonstrate an RNA species with a nucleotide composition somewhat similar to that of the DNA in normal rat liver nuclei. The material isolated from rat liver nuclei was shown to be highly active with respect to stimulation of amino acid incorporation into protein by pre-incubated _E. coli_ ribosomes[44]. The RNA extracted from purified liver ribosomes demonstrated a very low template activity. In fact, it was calculated to be no higher than 1-2% of the total ribosomal extract.

Up to this time, there was no study which related the early labeling ability of the RNA species to the protein synthesizing stimulating capacity. Such an experiment was reported by DiGirolamo et al.[45] The sedimentation pattern of pulse labeled RNA was investigated in rat liver nuclei and cytoplasm. In parallel studies, biologic activity was measured as defined by capacity to stimulate amino acid incorporation by a protein synthesizing system derived from _E. coli_. Comparing the sedimentation of the stimulatory RNA with that which takes up label at an early time after a pulse of C^{14}-orotic acid, it was noted that the patterns were strikingly similar in microsomal RNA. The largest concentration of both stimulatory and early-labeled material was found in the 18 S region. The nuclear patterns of stimulatory and early-labeled RNA, although more heterogeneous than the cytoplasmic

patterns, were also found to be similar. The proportion of early-labeled and of biologically active material relative to total RNA was considerably higher in the nuclear than in the microsomal fraction. Such a high proportion of messenger-RNA in liver nuclei, then, could account for the fact that the base composition of nuclear RNA differed from that of cytoplasmic and resembled more closely that of the DNA of the cell.

While a convenient relationship between pulse-labeling and template activity was demonstrated by DiGirolamo[45], it was soon evident that the template activity of the phenol-extracted RNA could be shown to sediment at remarkably distinct sedimentation constants depending upon the conditions of the extraction procedure. Brawerman et al.[46] illustrated that the material active in stimulating amino acid incorporation in normal rat liver and in rabbit reticulocytes was concentrated in the 18 S region when extracted at 4 degrees. In contradistinction to this, RNA extracted at 30 degrees was shown to concentrate most of its template activity in the 28 S region. This latter heavier activity peak was felt to represent a complex formation between template and ribosomal RNA. Furthermore, it was hypothesized that this complex formation resulted from the unmasking of reactive sites either on template RNA or on the ribosomal RNA during the heated phenol extraction. The implication which arose from this consideration was that such sites could account for the binding of template RNA to ribosomes within the cell.

Finally, adding to the confusion surrounding several of the characteristics

of M-RNA as described in the recent literature, Hadjivassiliou and Brawerman[47] reported on the occurrence of a template RNA and a DNA-like RNA representing two distinct entities in normal rat liver. An RNA fraction with high template activity was isolated from a rat liver nuclear pellet. This RNA species had a low G-C content which is characteristic of mammalian DNA and a high A/U ratio which was subsequently manifested in the pulse labeled RNA. In any event, this RNA seemed to be quite worthy of the status of messenger RNA. However, a more exhaustive study of this species revealed a complete separation of the low G-C RNA from the material with the template activity. The low G-C RNA appeared to sediment in the 10 S region and was characterized by a high A and low U content. The components rich in template activity, on the other hand, demonstrated a base composition closer to that of ribosomal RNA. The authors concluded that the 10 S RNA, without template activity, probably corresponded to chromatin RNA and was not a messenger for protein synthesis. Because of its close association with DNA, they speculated that it might conceivably have a regulatory function in the expression of genetic information.

The discovery of messenger RNA and the concept of the control of protein synthesis by repression has laid the groundwork for the understanding of the biochemistry of information transfer. The knowledge serving to functionally characterize M-RNA arose largely from bacterial systems. Following closely in the footsteps of this research, the elucidation of RNA with template activity invaded

the complexity of mammalian tissue. Reports quickly confirmed the presence of a rapidly-labeled, biologically active, DNA-like RNA in one mammalian tissue after another. Recently, the routine, almost glib acceptance of another example of M-RNA was put sharply to question. The significance of these periodic upheavals, I believe, is to prevent one from becoming so entwined in the experimental dogmatism of the moment so as to lose sight of the individual trees in the forest of mass-produced knowledge.

The present work was initiated with the intention of providing further evidence on the nature of messenger RNA. The ability of the liver to regenerate in response to partial hepatectomy provides a valuable comparative system. Thus, the heightened protein synthetic activity with the obligatory synthesis of different kinds of protein needed to restore lost parenchyma in the regenerating liver provides an experimental situation wherein comparison of template activity in normal and regenerating liver is possible.

This research concerns the overall template activity found in normal and regenerating livers, the size distribution of the messenger RNA molecule, and the rate of synthesis of the nuclear messenger RNA.

EXPERIMENTAL

The animals used in these experiments were Sprague-Dawley white rats weighing 300-400 grams. In all experiments, the animals were killed by decapitation and were allowed to exsanguinate.

I. Subtotal Hepatectomy Procedure - adapted from Higgins and Anderson[48].

Non-fasted animals were given ether anesthesia. A mid-line subxiphoid abdominal incision was performed and the large median lobe and left lateral lobe of the liver were delivered through the incision. The lobular base was ligated with 0 braided silk and the liver excised. In this manner, 65-75% of the total liver was removed, leaving within the peritoneal cavity the right lateral lobe and the caudate lobe. The wound was closed in two layers with 4-0 dermalon - the peritoneum and abdominal musculature approximated in one layer and the integument in the second. There was no special post-operative care, the animals being allowed food and water *ad libidum*. Regeneration time varied as stated.

II. Fractionation Procedures.

(a) Whole Cell Preparations: pH and temperature variability.

The rat liver was minced rapidly in ice-cold 10% sucrose solution. The tissue was homogenized in 5 volumes of 0.1 M Tris buffer pH 7.6 in an all glass tissue homogenizer in the cold. An equal volume of 80% ice-cold phenol was added. The RNA was extracted by constant stirring of the phenol mixture for 60 minutes either at room temperature or at higher temperatures as indicated. After extraction, the mixture was centrifuged for 30 minutes at 17,000 xg. The

aqueous supernatant layer was removed. The extraction procedures were carried out by thorough mixing of the gel interphase with the phenol base. An amount of 0.1 M Tris at the required pH equal to the volume used previously was added. The RNA in this mixture was extracted for 60 minutes, at the indicated temperature, centrifuged at 17,000 xg, and the aqueous supernatant layer removed. The RNA was precipitated from these aqueous extracts by addition of 2.5 volumes of 95% ethanol and 0.1 volume of 10% NaCl and storage at 4 degrees for 24 hours. The precipitates were collected and dissolved in a small volume of ice-cold water and adjusted with NaCl to a concentration of 10%. Storage at 4 degrees for 1-2 days brought about the precipitation of high molecular weight RNA. This was recovered by centrifugation. When needed, the RNA soluble in NaCl was obtained from the supernatant by precipitation with 2 volumes of ethanol. For the incorporation experiments, the pellets were freed of contaminating NaCl by suspension in ice-cold 66% aqueous ethanol and recentrifugation. The well-drained pellets were dissolved in water. All of the RNA preparations were stored in the deep-freeze.

(b) Modification of original extraction procedure - Separation of nuclei from cytoplasm.

In these procedures, all operations were carried out at 4 degrees C. The rat liver was minced rapidly in 10% sucrose solution. The tissue was disrupted in a teflon homogenizer in 10 volumes of a solution containing 10% sucrose, 0.05 M Tris pH 7.6, and 0.003 M $CaCl_2$. The homogenate was then filtered through

double thickness gauze to remove cellular debris. The material was centrifuged at 500 xg. The supernatant was considered the cytoplasmic fraction; the pellet, the crude nuclear fraction. To the supernatant was added 1/20 volume of 1.0 M Tris pH 9.0; a volume of ice-cold phenol equal to the supernatant and Tris buffer was then added and stirred gently at 4 degrees for 30 minutes. The remainder of the cytoplasmic extraction procedure was identical to that for the whole cell preparation.

The crude nuclear pellet obtained above was suspended in 4 times the volume of the original liver weight in a solution containing 75% sucrose, 0.01 Tris pH 7.6, 0.0015 $CaCl_2$. The nuclear material was then homogenized by hand in a teflon homogenizer and centrifuged at 50,000 xg for 45 minutes in the Spinco. The purified nuclear pellet was either stored in the freezer or subjected to phenol extraction as follows.

The pellet was suspended in 10 volumes of 0.01 M Tris pH 7.6 buffer and homogenized by hand in a teflon homogenizer. Aliquots of this homogenate were taken and 0.1 volume of 1.0 M Tris at pH 7.6, 8.5, 9.0, or 9.5 was added. A volume of ice-cold phenol, equal to the volume of the homogenate and buffer, was added and allowed to stir gently for 15 minutes at 4 degrees. The suspension was then centrifuged at 17,000 xg for 30 minutes and the supernatant removed. The phenol-gel mixture was re-extracted with 0.1 M Tris at the same pH, centrifuged, and the supernatant removed. The two supernatants were then treated individually

with 95% ethanol and 10% NaCl, aqueous NaCl solution, washed in 66% ethanol, and dissolved in water as described in (II-a). The final solution was saved for RNA analysis (Orcinol reaction)[49] and DNA analysis (Burton reaction)[50].

(c) High pH fractionation method of nuclei and cytoplasm.

The rat liver was disrupted in a teflon homogenizer in 10 volumes of a solution containing ice-cold 10% sucrose, 0.05 M Tris pH 7.6, 0.003 M $CaCl_2$. The homogenate was filtered through double thickness gauze and centrifuged at 500 xg. The supernatant was gently aspirated without disturbing the crude nuclear pellet.

To the supernatant - considered the cytoplasmic fraction - was added 1/20 volume 1.0 M Tris 7.6 and enough ice-cold 80% phenol to match the volume of homogenate and buffer. This mixture was first extracted at 4 degrees for 10 minutes and then divided into two aliquots, one extracted for 30 minutes at 4 degrees and the other, for 30 minutes at 38 degrees. The material was centrifuged for 30 minutes at 17,000 xg and the supernatants removed and treated in the usual manner (II-a). The phenol-gel mixture from the two aliquots was combined and thoroughly mixed. An equal volume of 0.1 M Tris pH 7.6 buffer was added, and the suspension stirred at 38 degrees for 30 minutes. Centrifugation at 17,000 xg for 30 minutes was effected; the supernatant was withdrawn and discarded. The phenol-gel mixture was re-extracted at 33 degrees and the supernatant withdrawn and discarded, thus removing all low pH cytoplasmic RNA. The phenol-gel mixture was re-extracted once more

with 0.1 M Tris pH 8.3 at 38 degrees. This served as the high pH cytoplasmic RNA. This procedure allowed the isolation of the following cytoplasmic RNA fractions:

1. cold extracted - pH 7.6 - ribosomal RNA
2. 38 degree extracted - pH 7.6 - ribosomal RNA
3. 33 degree extracted - pH 8.3 - high pH RNA

The crude nuclear pellet was suspended in 10 volumes of a solution composed of 75% sucrose, 0.01 M Tris pH 7.6, 0.002 M $CaCl_2$, and homogenized in a teflon homogenizer. The homogenate was centrifuged at 45,000 xg for 60 minutes and the purified nuclear pellet retained. The nuclei were carefully suspended in a volume of ice-cold 0.1 M Tris pH 7.6 equivalent to 4 times the original weight of the liver. A matching volume of ice-cold phenol was added and the mixture stirred at 4 degrees for 30 minutes. The suspension was centrifuged at 17,000 xg for 30 minutes and the supernatant treated in the usual manner. The phenol-gel interphase was well suspended in an equal volume of distilled water and stirred in a 38 degree water-bath for 5 minutes. 1.0 M Tris pH 8.3 buffer in an amount equivalent to one-tenth the volume of the distilled water was subsequently added and the stirring continued at 38 degrees for 30 minutes. The pretreatment of the extraction mixture at 38 degrees was required to avoid extraction of DNA. The presence of the water prevented dehydration of the phenol-gel interphase, permitting efficient extraction of the high pH RNA. The suspension was centrifuged and the supernatant removed and treated in the usual manner. This procedure allowed the isolation of nuclear

RNA fractions:

1. cold extracted pH 7.6
2. 38 degree extracted pH 8.3

III. Zone Centrifugation.

3 mg of purified aqueous RNA solution (1.5 ml) was layered over 28 ml of a 5-20% linear sucrose gradient supplemented with 0.01 M Tris pH 5.1 and 0.02 M NaCl. After centrifugation in the SW 25 Spinco rotor at 25,000 rev/min for 11-13 hours, 1.5-2.0 ml fractions were collected through the bottom of the tube. Three volumes of 95% ethanol were added to each fraction, and 0.5 ml samples of the suspensions were diluted with 2 ml of water for absorbancy measurements at 260 mμ. The remainder of each fraction was kept overnight in the cold. The precipitates were collected by centrifugation, dissolved in water, and used for RNA analysis and template activity assay.

Smaller amounts of RNA (100-300 μg) were centrifuged in the SW 39 Spinco rotor at 35,000 rev./min. Fractions of 4 drops and 14 drops were collected alternately. To the 14 drop fraction, 3 volumes of ethanol were added and the RNA was allowed to precipitate in the cold. They were used for isolation of centrifugal components or template activity analysis. The 4 drop fractions were diluted with 1 ml of water and the absorbancy at 260 mμ was measured. When C^{14}-labeled RNA was used, 0.7 ml samples of these latter solutions were taken, after UV measurements, for radioactivity countings. In some experiments where the amount of

radioactivity in the RNA was low, alternate samples of 10 drops each were used instead of 4 and 14 drops.

IV. Assay for Template Activity.

The assays were carried out with pre-incubated 30,000 xg supernatant of *E. coli* S-30 as described by Brawerman et al.[44] The total volume of the incubation mixtures was 0.15 ml. The composition of the reactions, modified from that used by Nirenberg and Matthaei[35], was as follows: Tris buffer pH 7.8, 0.1 M, KCl 32 mM, NH_4Cl 20 mM; Mg $(acetate)_2$ 12 mM, phosphoenolpyruvate Na salt 4 mM, ATP Na salt 1mM, GTP 0.6 mM, beta-mercaptoethanol 1.8 mM; mixture of unlabeled amino acids (minus leucine) 17 μM each; *E. coli* transfer RNA 110 μg/ml, phosphoenolpyruvate kinase 20 μg/ml; L-leucine-C^{14} uniformly labeled 40 μcurie/μmole, 0.8 μcurie/ml. The *E. coli* S-30 prepared according to Nirenberg and Matthaei[35] was diluted with an equal volume of high speed supernatant from *E. coli*. The latter was prepared from non-preincubated S-30 by centrifugation at 100,000 xg for 2 hours, followed by overnight dialysis in the cold against 10 mM Tris pH 8.0 and 6 mM beta-mercaptoethanol. This dilution resulted in a considerable enhancement of the stimulation of amino acid incorporation by added RNA. The amount of diluted S-30 used per 0.15 ml reaction was 0.02 ml. The RNA samples to be tested were included in concentrations usually lower than 100 μg/ml. Since a certain amount of variability was observed in the stimulatory activities obtained in different experiments, a standard RNA preparation was usually run simultaneously

with the samples in order to relate results obtained from different experiments.

For the study of sedimentation patterns of material with template activity, the 14 drop fractions obtained by zone centrifugation, to which 3 volumes of ethanol had been added, were kept in the cold for at least 1 day to allow for precipitation of the RNA. After centrifugation at 2500 rev./min. for 20 minutes, the supernatants were removed and the tubes inverted and allowed to drain for about 1 hour in the cold to insure complete removal of the ethanol. This procedure permitted the quantitative precipitation of the RNA even when only a few µg were present. The precipitates were then dissolved in the appropriate amount of water and the components for the template activity assays were added to the tubes. In this manner, the assays were carried out in the same tubes in which the 14 drop fractions had been collected. The template activity was expressed as increase in µµ moles of leucine incorporated upon addition of the template RNA.

V. Pulse Labeling.

A 40 minute pulse of Orotic acid 6-C^{14} in a dose of 10 µcuries/100 rat grams was administered intra-peritoneally. The radioactivity was measured by spotting 20λ of the RNA solutions on filter paper disks. The disks, after being allowed to dry, were washed for 30 minutes in cold 5% TCA and then rinsed in ether-ethanol 3/1-v/v. Scintillation mix was added and the vials counted for 10 minutes.

VI. Analytical Procedures.

RNA analysis.

The purified RNA preparations were hydrolyzed in 0.5 N $HClO_4$ at 70 degrees for 20 minutes. The absorbancy of the hydrolysates at 260 mμ was used to determine the RNA content. The ratio of absorbancies A_{280}/A_{260} was used to obtain a rough estimate of the G-C content of the RNA samples[44]. The fractions after zone centrifugation were read directly in water using a value of 1.0 for the A_{260} solution of 45 μg/ml.

RESULTS

1. Effect of pH and Temperature on RNA Yields and Template Activity.

The original procedure for RNA extraction frequently led to heavy contamination of the RNA by DNA, especially at high pH's, and the presence of this DNA prevented the NaCl precipitation of the RNA. It was observed that slight heating of the phenol mixtures in the pH 7.6 buffer resulted in a considerable reduction in the amount of DNA obtained in the subsequent extractions at elevated pH.

In this experiment, 12 grams of normal liver were subjected to the RNA extraction procedure (II-a). Five aliquots of the liver homogenate were used and extracted either at room temperature or at elevated temperatures. Several fractions (IV, V) were sequentially re-extracted at higher pH's.

As can be noted in Table I, heating the phenol extraction mixture to 50 degrees increases the yield of RNA as compared to the room temperature extracted RNA. The bulk of the RNA is extracted at neutral pH, and re-extraction of the RNA fractions increases the yield only to a small degree. The template activity of the RNA increases with the increasing pH of the extraction mixture. The specific template activity also increases upon heating the phenol extraction mixture. The absorbancy ratios, A_{280}/A_{260}, which have been shown to be a measure of the G-C content of the RNA[44] are all in the range for ribosomal RNA. This indicates that the material responsible for the template activity probably represents only a minor portion of the total RNA since it has not influenced the G-C content significantly.

TABLE I

Effect of pH and Temperature on RNA Yields and Template Activity

Fraction Number	Extraction pH	Extraction Temperature	A_{280}/A_{260}	Total mg RNA	Specific Template Activity
I	8.5	R.T.	.73	12.8	520
II	8.5	40/5"	.73	9.0	1140
III	8.5	50/5"	.75	21.4	1300
IV$_1$	7.6	R.T.	.74	15.6	560
V$_1$	7.6	50/5"	.74	22.9	1050
IV$_2$	7.6	R.T.	.75	3.0	
V$_2$	7.6	50/5"	.75	1.4	
IV$_3$	7.6	R.T.	.76	1.2	
V$_3$	7.6	50/5"	.73	0.64	
IV$_4$	8.5	R.T.	.73	0.56	1830
V$_4$	8.5	50/5"	.74	0.29	3370
IV$_5$*	9.0	R.T.	.73	1.25	
V$_5$**	9.0	50/5"	.77	0.28	3100
V$_5$	9.0	50/5"	.72	0.13	6050

* Fraction contained DNA

** Material soluble in 10% NaCl

Legend to Table I

Five aliquots obtained from a whole cell liver homogenate derived from 12 gms of normal liver were subjected to phenol extraction. Fractions I, II, III were extracted at pH 8.5, while fractions IV and V were extracted at pH 7.6. The homogenates were mixed with phenol at 0 degrees and then brought to the specified temperature as indicated in the third column. Fractions IV and V were re-extracted twice more under the original conditions, followed by a third extraction at pH 8.3 and a fourth at pH 9.0. The A_{280}/A_{260} readings - an expression of the G-C content of the RNA fractions - are listed in the fourth column. The yield in mg of RNA is listed and the specific template activity is reported in the last column.

R.T.: Room temperature.

40/5"; 50/5": 55 minutes of phenol extraction at room temperature and 5 minutes of extraction at either 40 degrees or 50 degrees C.

Specific Template Activity: Amount of stimulation per mg RNA.

II. Effect of Regeneration Time on RNA Yields per Gram of Tissue with Corresponding Specific Activities - Whole Cell Preparations.

An apparent progressive increase in the yield of RNA/gram of liver is obtained with increasing regeneration time. In this experiment, the 14 hour RNA tissue concentration is considerably lower than that for normal liver. The RNA tissue concentration exceeds that of normal liver by 48 hours of regeneration. The specific template activity of the 14 hour regenerating liver is inferior to that seen for normal liver, while the 72 hour regenerating liver's template activity exceeds that for normal liver.

This aspect of the research was not studied in detail and the conclusions must be tentative. However, the results of the following experiment also indicate that the specific template activity in the 72 hour-50 degree regenerating liver exceeds that for normal liver extracted at 50 degrees C, thus lending credence to these results.

TABLE II

Effect of Regeneration Time on RNA Yields per Gram of Tissue With Corresponding Specific Activities - Whole Cell Preparations

Liver	Extraction pH	Extraction Temperature	RNA/gm Liver	Specific Template Activity
14 hrs	8.0	50/5"	1.2	145
24 hrs	8.0	50/5"	2.2	
48 hrs	8.0	50/5"	2.96	
72 hrs	8.0	50/5"	2.9	400
normal	8.0	50/5"	2.5	280

Legend

Regenerating and normal liver was subjected to whole cell phenol extraction at pH 8.0 for 55 minutes at room temperature and then for 5 minutes at 50 degrees C. The fractions were assayed for their RNA content by U-V analysis and the yields of RNA expressed per gram of liver used. Several of the fractions of RNA were assayed for template activity. The results are listed in the last column.

III. Effect of RNA Concentration on Specific Activity.

Template activity in whole cell RNA preparations extracted at room temperature or at 50 degrees for 5 minutes was measured. The results for normal and 72 hour regenerating liver tissue are recorded in Table III.

A linear relationship to different doses of template RNA is indicated by a constant specific template activity value. From this experiment, it is noted that only in the heat-extracted fractions is there constancy of specific activity; thus a linear relationship is maintained upon doubling of the amount of template RNA. This behavior could be due to the presence of an inhibitor in the cold-extracted RNA. This possibility, however, was not further explored.

TABLE III

Effect of RNA Concentration on Specific Activity

Liver	γ/ml RNA	Extraction pH	Extraction Temperature	Specific Template Activity
normal	135	8.5	R.T.	1110
normal	270	8.5	R.T.	580
normal	135	8.5	50/5"	590
normal	306	8.5	50/5"	552
72 hrs	177	8.0	R.T.	1130
72 hrs	354	8.0	R.T.	390
72 hrs	131	8.0	50/5"	810
72 hrs	262	8.0	50/5"	780

Legend

Whole cell RNA from normal liver extracted at pH 8.5 at both room temperature and at 50 degrees for 5 minutes was obtained. Whole cell RNA from 72 hour regenerating liver, extracted at pH 8.0 at both room temperature and at 50 degrees for 5 minutes was also obtained. A single and double dose of RNA from each fraction was assayed for template activity. The specific template activity is listed in the last column.

IV. Sedimentation Characteristics of Rat Liver Cytoplasmic RNA.

The extraction procedure (II-b) was applied to both normal and 24 hour regenerating liver in this experiment. The sedimentation pattern shows the customary two ribosomal components with sedimentation values set at 28 S and 18 S for both fractions. The amino acyl transfer RNA has been removed in the 10% NaCl precipitation step. The material with the template activity appears in two well defined zones. The pattern in Fig. I-a demonstrates that the major portion of activity resides in the 18 S region for normal liver. This is in contrast, Fig. I-b, to the 24 hour regenerating liver where the bulk of the template activity is in the 28 S region. The absorbancy ratios 280 mμ/260 mμ, which are a measure of the G-C content[44], are plotted for each RNA fraction. The results indicate that for both regenerating and normal liver the 18 S ribosomal components contain less G and C than does the 28 S component. This difference has been noted by others[51, 52, 46].

It must be stressed that the template activity profiles obtained for regenerating liver did vary. In several experiments the predominant template peak appeared in the 18 S region instead of in the 28 S region as presented in Fig. I-b. The explanation for this variability is not readily apparent. While it is known that the sedimentation characteristics of the template material in normal liver can be altered by increasing the temperature of the RNA-phenol extraction[51, 52, 46], the same rationale can not be applied to shifts in template activity isolated at the same temperature - in this case 4 degrees C.

FIG. 1—a

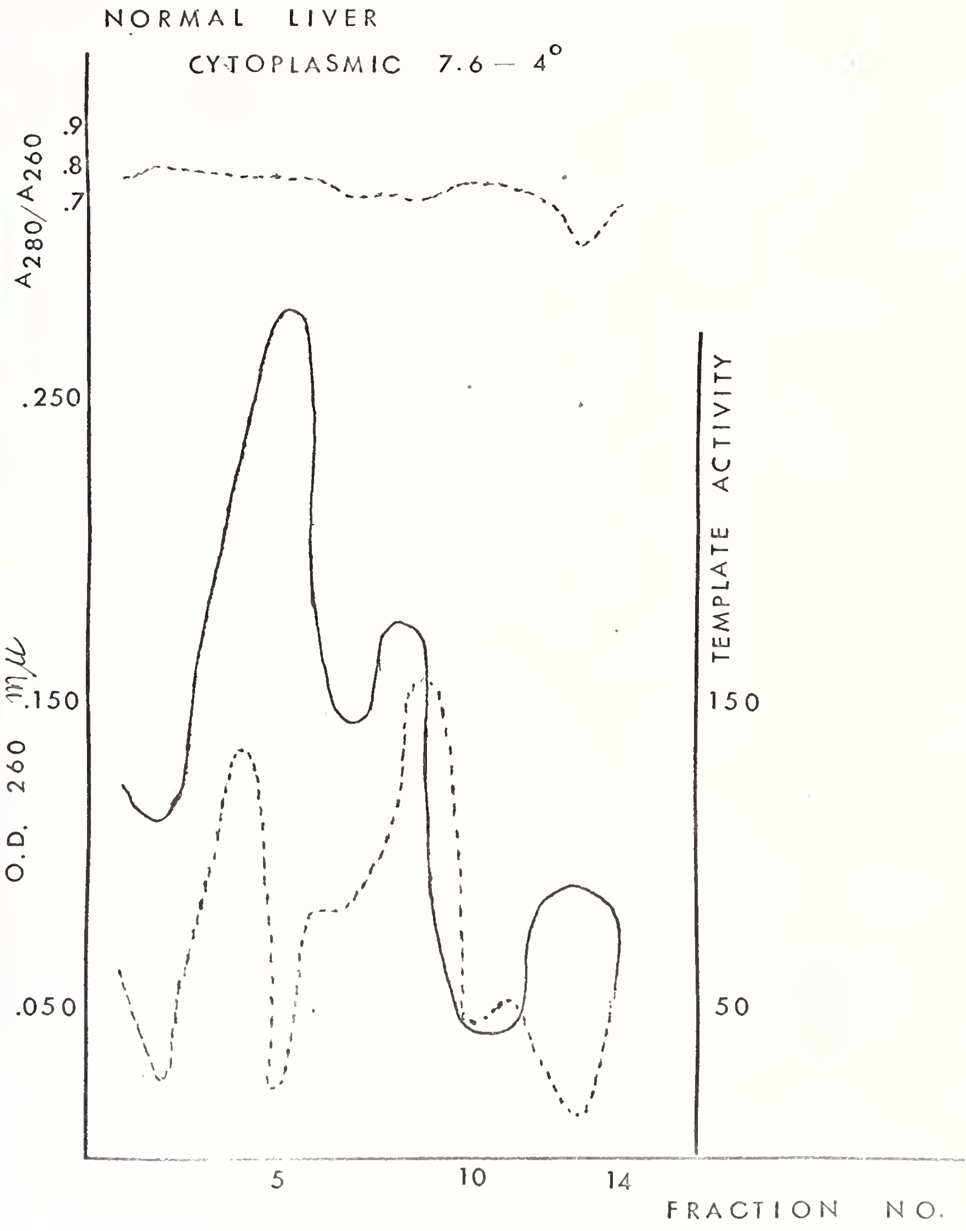
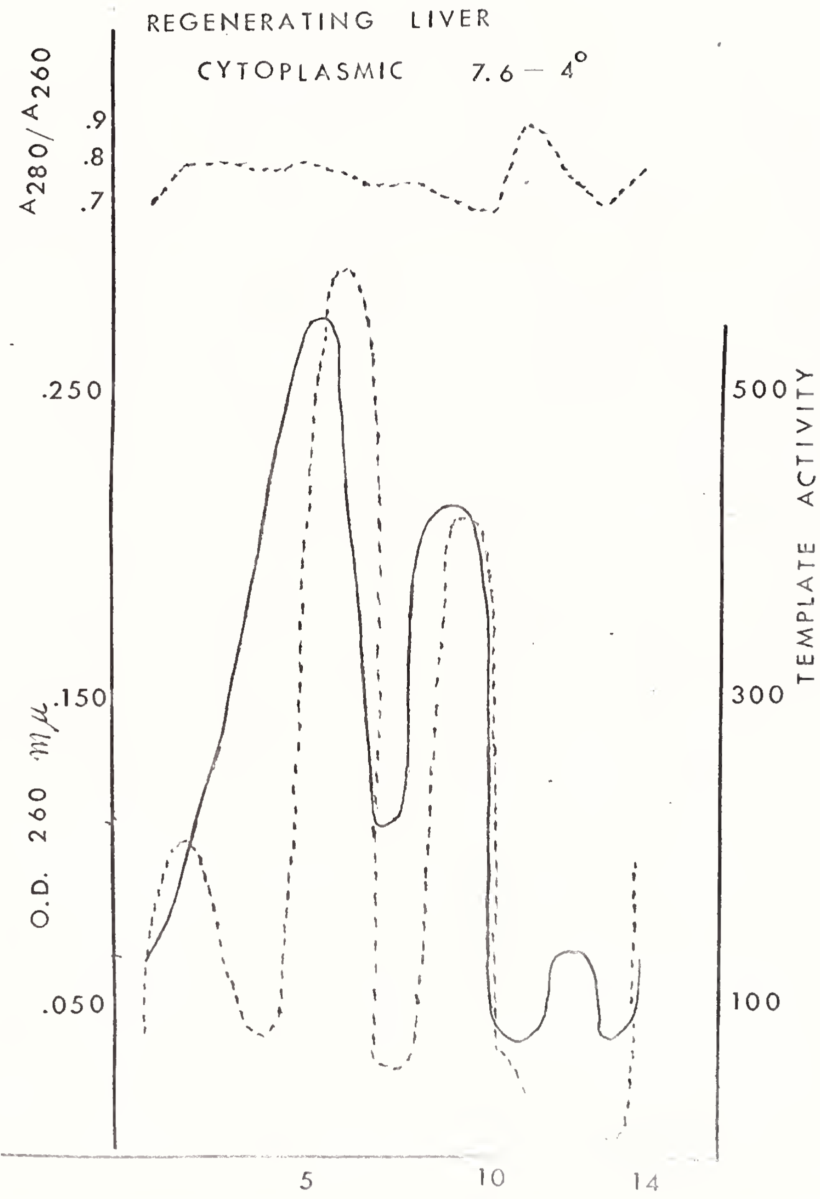

NORMAL LIVER
CYTOPLASMIC 7.6 — 4°
A_{280}/A_{260}
.9
.8
.7
.250
.150
.050
O.D. 260 mμ
TEMPLATE ACTIVITY
150
50
5
10
14
FRACTION NO.

FIG. 1— b


REGENERATING LIVER
CYTOPLASMIC 7.6 — 4°
A_{280}/A_{260}
.9
.8
.7
.250
.150
.050
O.D. 260 mμ
TEMPLATE ACTIVITY
500
300
100
5
10
14

Legend Figure I - A and B

Zone Centrifugation of Rat Liver Fractions

(A) 3 mg of cytoplasmic RNA obtained from normal liver extracted at pH 7.6 - 4 degrees, was layered over a 5-20% linear sucrose gradient in the presence of 0.02 M NaCl. Centrifugation time was 11 hours.

(B) 3 mg of cytoplasmic RNA of 24 hour regenerating liver extracted at pH 7.6 - 4 degrees C was treated as above.

Template activity (----) is expressed as the specific template activity - i.e. the amount of stimulation per mg RNA per fraction. Total RNA (———) is expressed as the absorbancy measurements at 260 mμ prior to precipitation with ethanol. Absorbancy ratios (- - --) are expressed as the ratio of the absorbancy measurements at 280 mμ and at 260 mμ.

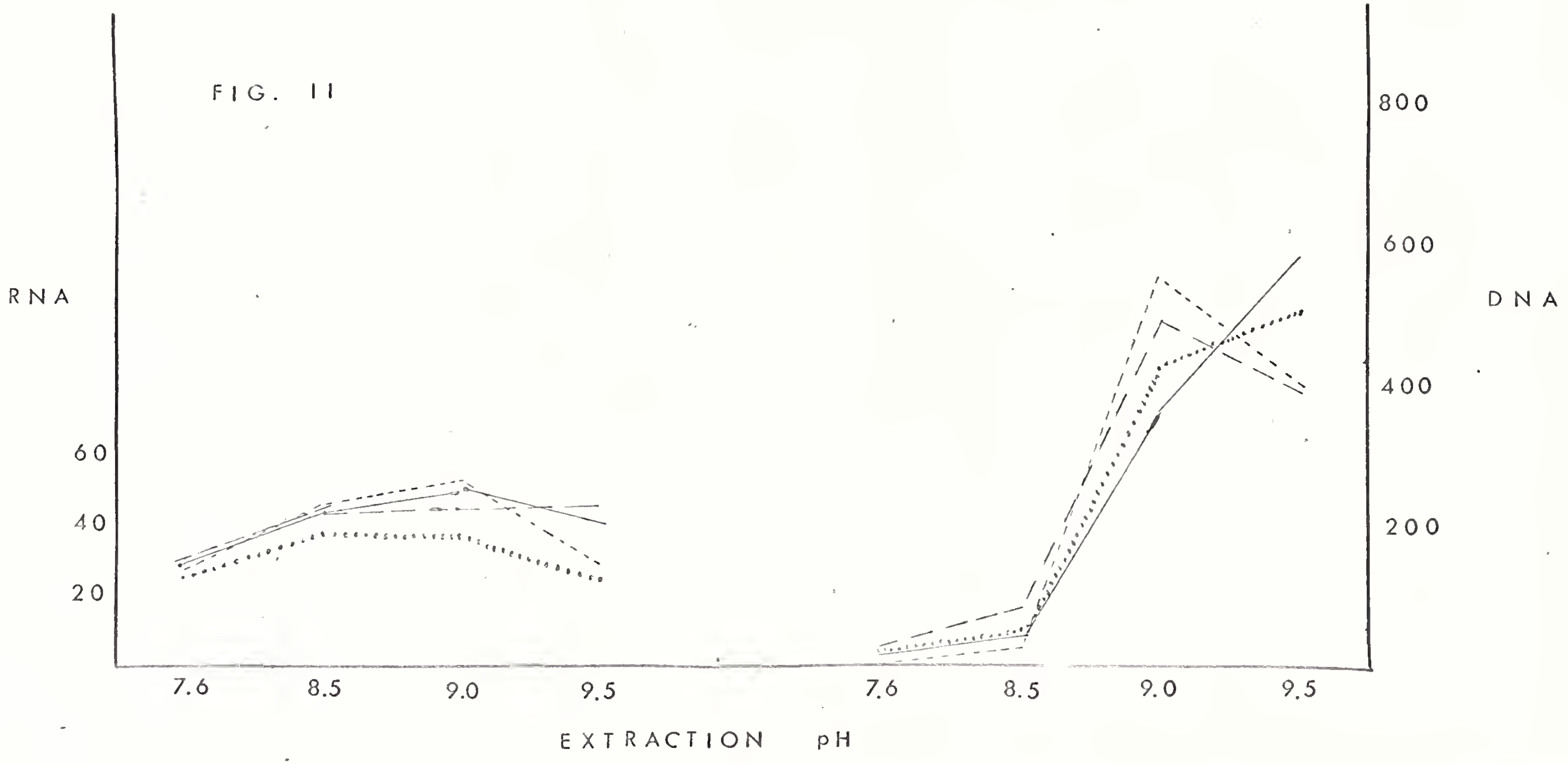
V. Extraction of Nuclear Pellet.

In the process of attempting to isolate RNA free of contamination of DNA, it was observed that the DNA in regenerating liver appeared to be more easily extractable than in normal liver. On this premise, a study was undertaken to extract quantitatively DNA and RNA from nuclear pellets at various pH's in order to define those conditions most amenable to the extraction of high pH RNA without DNA.

Crude nuclear pellets obtained from normal, 24, 48, and 72 hour regenerating livers were assayed for their total RNA and DNA contents. The nuclear homogenate was divided into aliquots and extracted twice in the cold at different pH's. The RNA and DNA were measured by the Orcinol[49] and Burton[50] diphenylamine methods respectively. Cumulative values for each of the two extractions are plotted in Fig. II.

It was found that at pH 9.0, there was a tendency for the near total extraction of DNA in the 48 and 72 hour regenerating liver pellets, whereas in the 24 hour regenerating sample and in normal liver, less DNA was extracted. Because of the incomplete ability of this technique to separate nuclear RNA from DNA at high pH's, a method utilizing both elevated pH and elevated temperatures was developed. This method is described in detail in the Experimental section II-c.

FIG. 11


RNA
60
40
20
DNA
800
600
400
200
7.6
8.5
9.0
9.5
7.6
8.5
9.0
9.5
EXTRACTION pH

Legend Figure II

Nuclear pellets were obtained from normal and 24, 48, and 72 hour regenerating livers as described in the Experimental section II-b. Each pellet was homogenized and divided into 4 aliquots. A Tris buffer at pH 7.6, 8.5, 9.0, or 9.5 was added to each aliquot. Ice-cold phenol was added, and each aliquot was extracted twice at 4 degrees C for 15 minutes. The Orcinol reaction[49] for RNA analysis and the Burton reaction[50] for DNA analysis were performed on all solutions. The graphs represent the cumulative yields of DNA and RNA obtained by summating the yields from the first and second extractions. The DNA is expressed as μg of DNA and the RNA as μg/mg DNA extracted.

———— 24 hour regenerating liver

— — 48 hour regenerating liver

- - - - 72 hour regenerating liver

· · · · · normal liver

VI. Comparison of Pulse-Labeling in Normal and Regenerating Liver.

In the high pH fractionation of normal liver[53], it was found that the bulk of the RNA was extracted from the cytoplasm at neutral pH. This was considered to be ribosomal RNA. The cytoplasmic 8.3 fraction contained high template activity and was labeled with a moderate amount of C^{14}-orotic acid. This fraction was considered to be rich in messenger RNA. Two nuclear fractions were also examined. The nuclear 7.6 fraction was characterized by a low amount of template activity and a moderate amount of radioactivity. This fraction was presumed to represent nucleolar RNA. The high pH nuclear RNA (8.3) was found to be rich in template activity and contained most of the radioactivity. This was interpreted to represent chromatin RNA. With these data established for normal liver, similar experiments were performed to determine whether or not there would be any significant differences in the amount of RNA, labeling, or template activity in the RNA extracted from regenerating liver. In the following experiments, the differences and similarities of normal and regenerating liver are reported.

20λ of the RNA solutions, subjected to the high pH fractionation procedure (II-c) obtained from normal and 24 hour regenerating livers, was assayed for radioactivity. The results in Table IV are expressed in counts per minute per mg RNA. Appointing the specific radioactivity of the nuclear 8.3 fraction as 100%, the other fractions may be compared to one another by expressing

their cpm/mg RNA as a percentage of the nuclear 8.3 value. Since both the normal and regenerating nuclear 8.3 RNA fractions have about the same radioactivity, all percentages in both experiments are comparable. Also included in the table are the mgs of RNA and percents of the total yield of RNA as represented by each fraction.

The highest specific radioactivity is found in the high pH nuclear RNA for both normal and regenerating liver. This high specific radioactivity is contrasted to the extremely small percentage of the total yield of RNA that is represented by the high pH nuclear fractions.

The nuclear 7.6 fraction for regenerating liver has over three times the specific radioactivity than does the corresponding normal liver fraction. If the labeled material, which is located almost exclusively in the 28 S and heavier region (see Fig. V), represents newly synthesized ribosomal RNA, then the higher specific radioactivity of regenerating liver could be explained by the increased demand for ribosomes in the regenerating process.

It is of interest to note that in normal cytoplasmic 8.3 liver, the specific radioactivity is over three times as great as that seen in the regenerating cytoplasmic 8.3 fraction. This difference is contrasted to the approximate 5 fold increase in RNA yield seen in the regenerating fraction. If the 18 S component (see Fig. IV) is the material which contributes to the higher yields of RNA and yet is not labeled to any considerable extent, then the low specific activity but

high yields of cytoplasmic 8.3 regenerating RNA are reconcilable.

The cytoplasmic 7.6 fraction has a rather low specific radioactivity. However, the bulk of the RNA is extracted at this pH. This RNA is interpreted to be in large part ribosomal RNA. The yield of RNA extracted at pH 7.6 is substantially greater for regenerating liver. This may be another manifestation of the increased demand for ribosomal RNA in regenerative tissue.

TABLE IV

Comparison of Pulse-Labeling in Normal and Regenerating Liver

NORMAL LIVER

Fraction	RNA mg Extracted	% Total RNA Yield	Specific Radioact.	% Specific Radioact.
cyto. 7.6	45.0	93.2	4000	0.81
cyto. 8.3	0.9	1.8	50400	10.3
nuc. 7.6	1.3	2.7	46700	9.5
nuc. 8.3	1.1	2.3	490000	100.0
TOTAL	48.3			

REGENERATING LIVER

Fraction	RNA mg Extracted	% Total RNA Yield	Specific Radioact.	% Specific Radioact.
cyto. 7.6	64.0	90.0	8600	1.81
cyto. 8.3	4.4	6.1	14700	3.1
nuc. 7.6	1.1	1.5	139000	29.3
nuc. 8.3	1.75	2.4	474000	100.0
TOTAL	71.25			

Legend to Table IV

RNA was extracted from 26 grams of normal liver and 25 grams of 24 hour regenerating liver according to the procedure outlined in section II-c of the Experimental. In addition, the rats were exposed to a 40 minute pulse of C^{14}-orotic acid. 20 of the RNA solutions was applied to filter paper disks, washed with 5% TCA and rinsed in ether-ethanol solution. Scintillation mix was added and the vials counted for 10 minutes. The specific radioactivity (counts/minute/mg RNA) for each fraction is listed, along with the percent of the total specific radioactivity. The extraction yields in mg of RNA and the corresponding percent of the total yields are recorded for each fraction.

VII. Sedimentation Characteristics of Regenerating Rat-Liver RNA Fractions.

The RNA was submitted to the high pH fractionation procedure outlined in section II-c of the Experimental. The several fractions underwent gradient centrifugation, radioactivity counting, and template assay. In all cases, the liver was allowed to regenerate for 24 hours and a 40 minute pulse of C^{14}-orotic acid was used. All references in this section to normal liver for comparative purposes are taken from the work of Hadjivassiliou and Brawerman[53]. In their experiments, normal liver was subjected to the identical procedures as reported here for regenerating liver.

Cytoplasmic 7.6 - cold Fig. III-a

The U-V pattern shows the customary two ribosomal components with sedimentation values of approximately 28 S and 18 S. The material with the template activity appears in 2 zones, corresponding in large part to the ribosomal peaks. The more active template peak is localized in the 18 S region. The radioactivity appears to coincide with the ribosomal peaks; however, there is significantly more pulse-labeling in the 18 S zone. This is in accord with many reports that the newly synthesized 18 S ribosomal RNA appears in the cytoplasm prior to the 28 S RNA[54, 39]. The labeled material near the top of the gradient may represent degraded pulse-labeled fragments or it may correspond to the light peak observed in the U-V pattern in the same region of the gradient. It is unlikely that it represents transfer RNA since the 10% NaCl purification step leaves the

S-RNA in the supernatant. This material was not further studied.

Similar patterns for U-V absorption, pulse-labeling and template activity were found for normal liver RNA extracted under the same conditions.

Cytoplasmic 7.6 - 38 degrees Fig. III-b

The two ribosomal RNA peaks noted before in the cold-extracted pH 7.6 RNA are essentially unchanged. Likewise, the template activity patterns appear to be equivalent. It is of interest that the shift of template activity to the 28 S region of the gradient described for normal liver extracted at 39 degrees[51, 52, 46] does not occur for regenerating liver extracted under the same conditions - the postulated ribosomal-template complex not forming[46]. Whether this is due to experimental variability or whether this is indeed a valid property of regenerating liver can not be stated with certainty. It would appear, however, from other experiments that the latter is more likely.

Pulse labeling occurs almost exclusively in the 18 S region of the gradient where are also concentrated the template activity and the heavy 18 S ribosomal peak. There is a large upsurge of pulse labeling in the light region of this gradient as was observed in the cytoplasmic 7.6 cold fraction. This was also present in normal liver. The U-V absorption and pulse-labeling patterns for normal liver mimic those for regenerating liver. The template activity, however, has shifted to the heavier, 28 S region in the heat-extracted normal liver, consistent with previous reports[46, 47]. The apparent absence of the shift of template

activity and pulse labeling in regenerating liver does not allow any definite interpretations as to the nature of the pulse-labeled material. However, in normal liver, the shift of template activity is not followed by a parallel change in the pulse labeling. This would suggest that the RNA with pulse labeling and the template activity do not represent the same entity.

Cytoplasmic 8.3 - 33 degrees   Fig. IV

In this fraction, the U-V absorption pattern differs from the pH 7.6 cytoplasmic RNA. In place of the 2 major ribosomal components, there is only one major U-V peak located in the 18 S region which contains a small shoulder extending into the 10 S region of the gradient. A minor peak of U-V absorption is still evident in the 28 S zone, however. Contained within the large, sharply defined U-V peak is the template activity pattern. Lesser, ill-defined amounts of template activity are present in the heavier regions of the gradient. The pulse-labeled radioactivity builds to a broad peak which sediments in the same range as do the major U-V peak and template activity.

The patterns for normal liver are remarkably similar, with slight modification. Namely, the 18 S, U-V peak is smaller than its counterpart in regenerating liver. In addition, the material in the 10 S region, represented by a shoulder of U-V absorption forming the light border of the major U-V peak, is more prominent. This shoulder was investigated in greater detail in normal liver. It was found to represent polyadenylic acid.

Nuclear 7.6 - cold Fig. V

In the neutral pH nuclear RNA, the U-V absorption pattern is again different from any of the previous patterns. There are 3 well-defined peaks sedimenting at approximately 28 S, 18 S, and 5 S. The 28 S and 5 S peaks are more prominent.

The template activity does not assume a pattern sharply mimicing the U-V absorption peaks as it had in the cytoplasmic 8.3 RNA fractions, but now presents a more diffuse pattern with broad irregular elevations. The pulse labeling is also unique in that it appears as a solitary peak in the heavy region of the gradient. It was found to sediment consistently in the zone preceeding the 28 S U-V peak. This material probably represents precursor ribosomal RNA, consistent with findings that ribosomal precursors are heavier than 28 S[39, 55].

The pattern for normal liver is surprisingly different from that of regenerating liver. While there are the 3 peaks of U-V absorption as in normal liver, the ratio of 28 S/18 S material is greater in regenerating liver. The template activity is even more diffuse and patternless than was seen for regenerating liver. Most strikingly, the pulse labeling is localized in 2 large peaks, one slightly heavier than the 28 S U-V pattern, and the other encompassing a broad area in the light region of the gradient. It is of interest that in regenerating liver, the bulk of the radioactivity is localized in the heavy region, while in normal liver, the larger amount of radioactivity resides in the light region (18 S).

Nuclear 8.3 - 33 degrees Fig. VI

In the high pH nuclear fraction, the U-V absorption pattern demonstrates a broad, heavy peak (27-33 S) and a sharper, lighter peak (16 S). There is a third small peak in the light region of the gradient.

In the normal liver, the U-V absorption consists of 3 peaks as seen in the regenerating liver; however, the heavy peak is much less prominent.

In regenerating liver, the template activity is irregular with a series of elevations and one sharp peak of activity located in the 16 S region, coincident with the U-V absorption in that area. However, in normal liver, the template activity profile describes a neater pattern with the major concentration located in the 9-16 S region.

The bulk of the pulse labeling occurs in the heavy region for regenerating liver, while the predominance of pulse labeling is found in the 9-16 S region for normal liver.

From the study of base compositions in both normal and regenerating liver[56], the material in the heavy region of the gradient resembles ribosomal RNA, while the material in the light region (9-16 S) has a DNA-like base composition. Since there is little template activity in the heavy region as contrasted to large amounts in the 9-16 S region, it is thought that the heavy region represents ribosomal RNA and the light region M-RNA. In this context, the labeling patterns in normal and regenerating liver would support this view. In regenerating liver, most of the

radioactivity is located in the heavy region while in normal liver, most of the radioactivity is found in the light region (9-16 S). Therefore, it would appear that regenerating liver predominantly synthesizes ribosomal RNA and normal liver, predominantly messenger RNA.

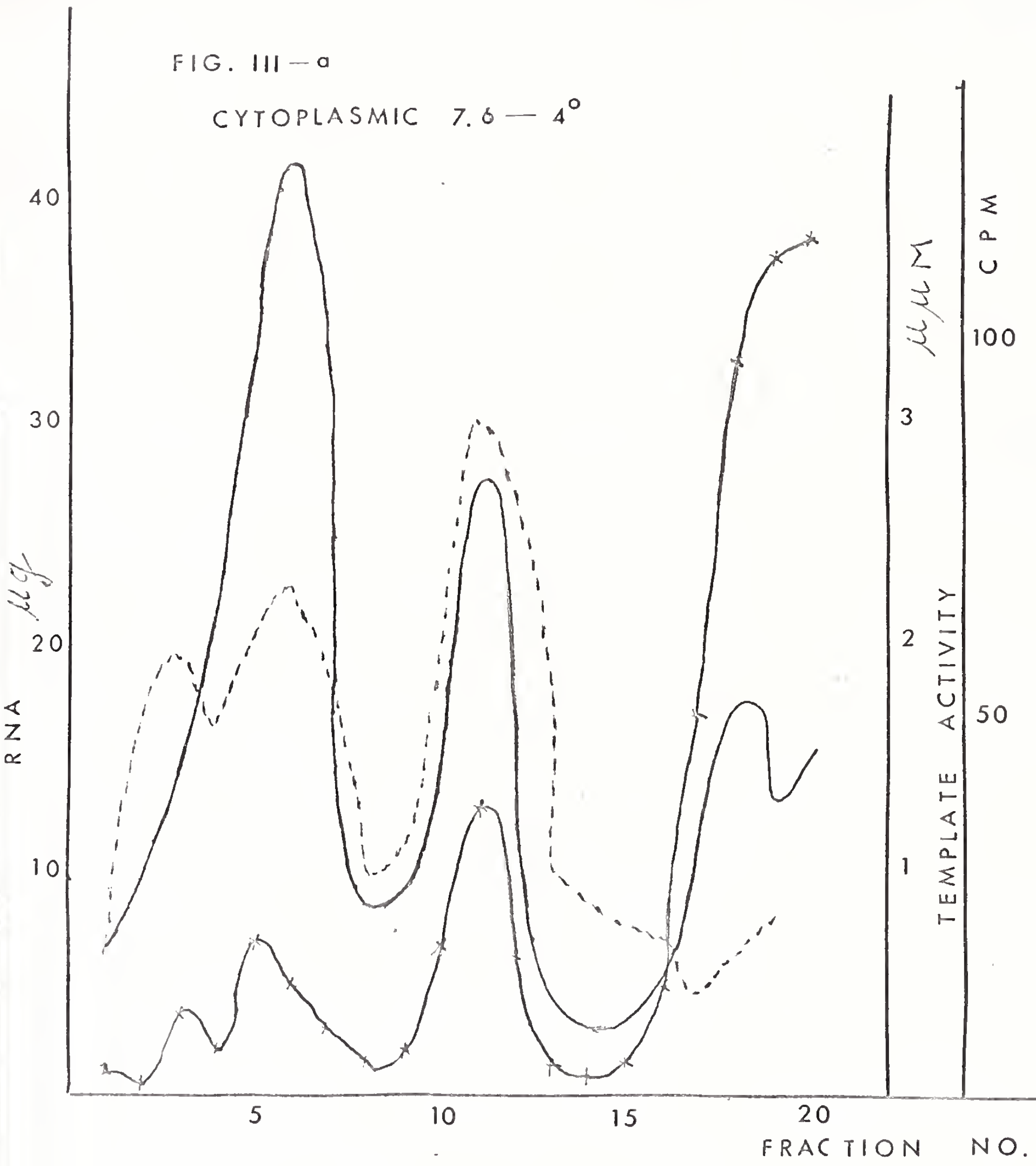
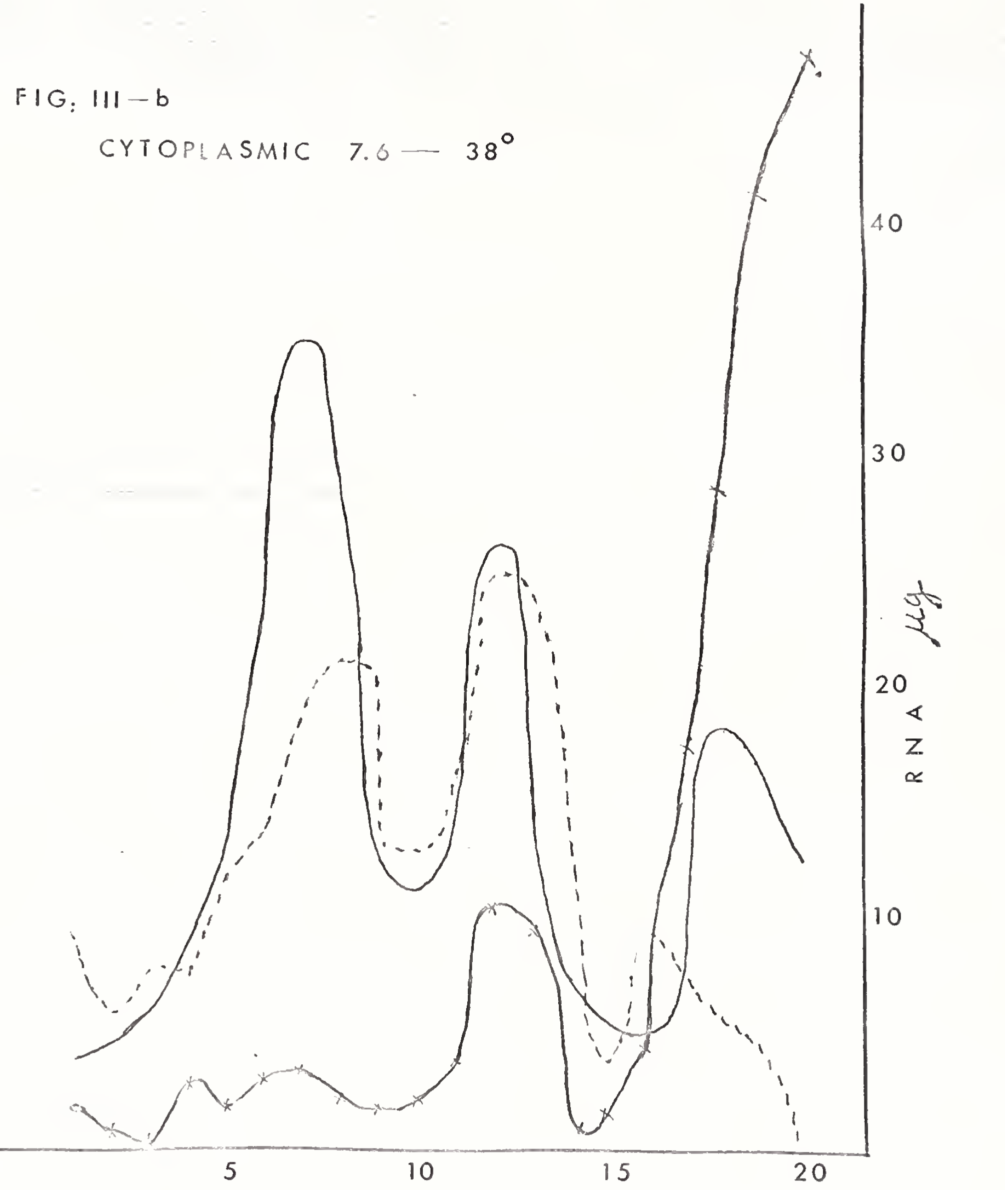


FIG. III — a

CYTOPLASMIC 7.6 — 4°



FIG; III — b

CYTOPLASMIC 7.6 — 38°

FIG. IV

CYTOPLASMIC 8.3

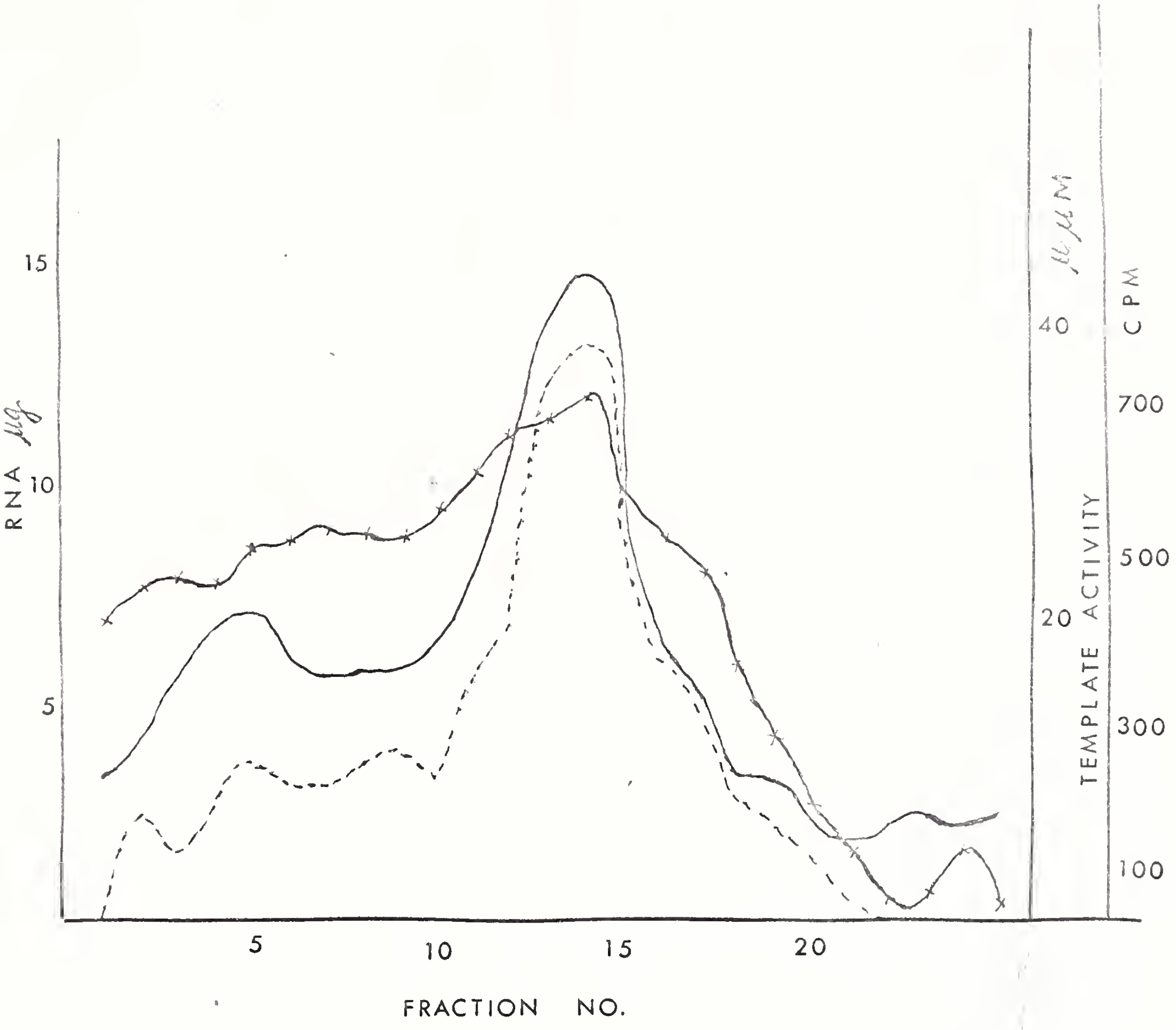


FIG. V

NUCLEAR 7.6

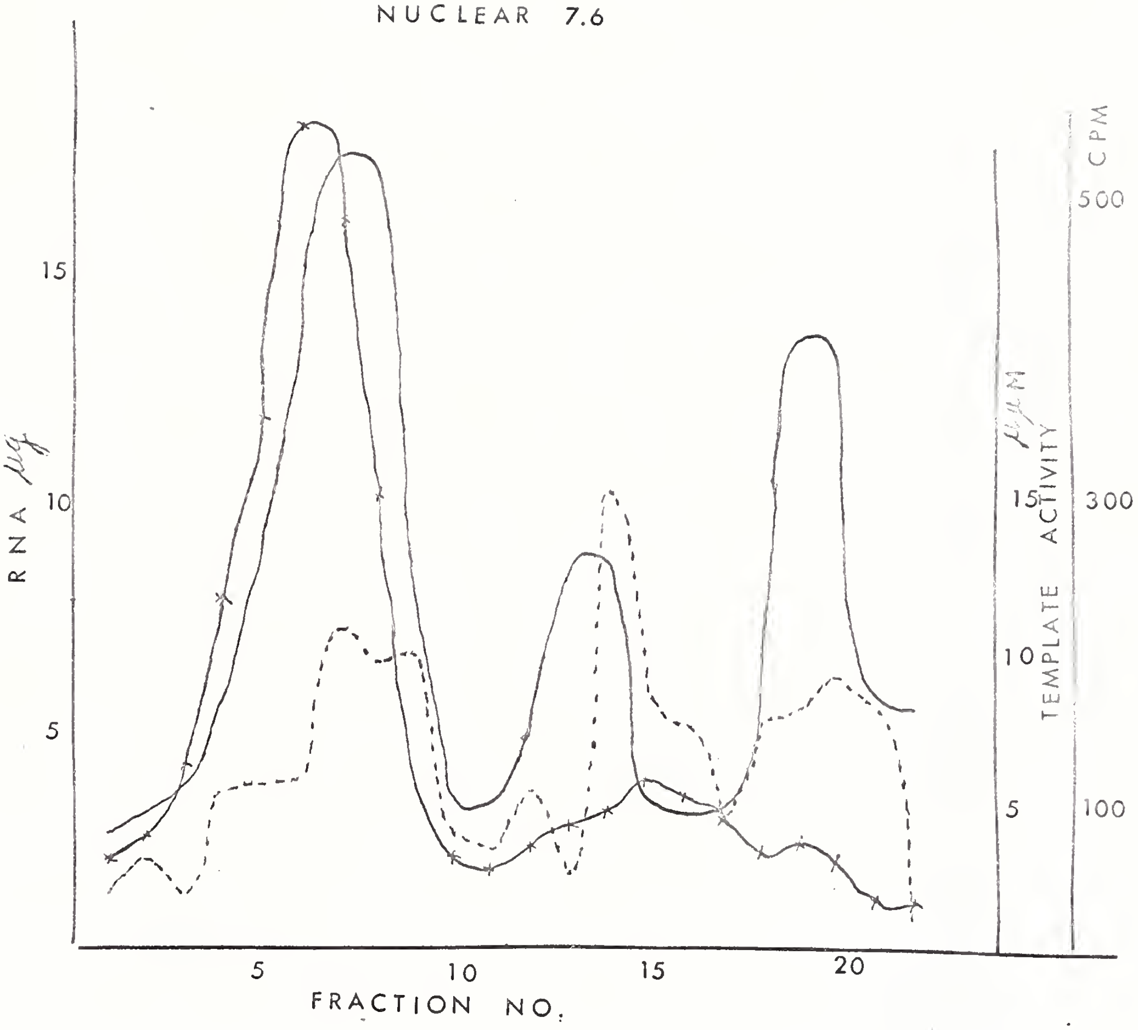
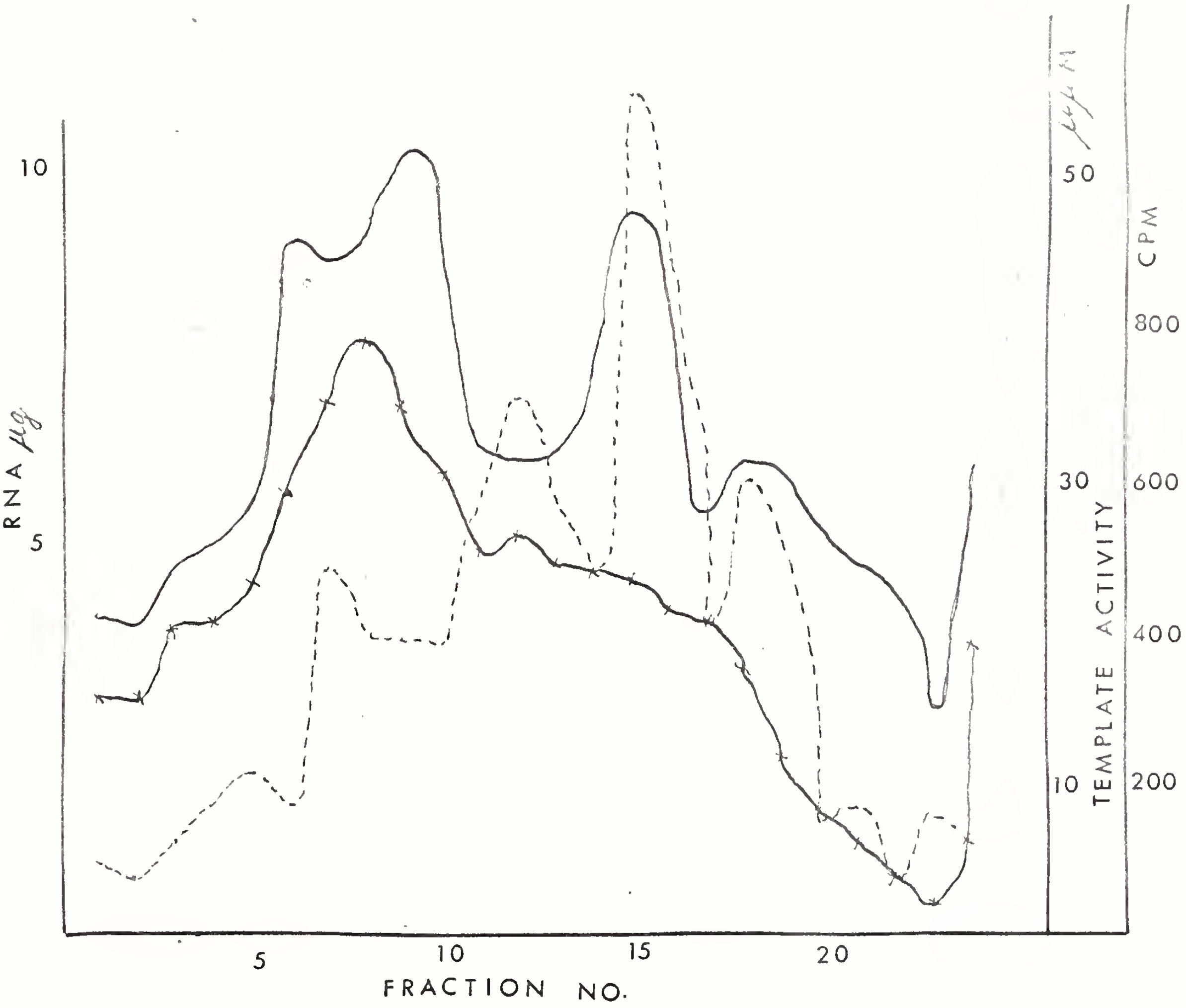


FIG. VI

NUCLEAR 8.3



Legend Figures III-VI

Zone Centrifugation of Regenerating Rat Liver RNA Fractions

Using the high pH fractionation procedure, RNA was isolated from 24 hour regenerating livers which had received a 40 minute pulse of C^{14}-orotic acid. The following fractions were obtained:

cytoplasmic 7.6 - cold

cytoplasmic 7.6 - 33 degrees

cytoplasmic 8.3 - 33 degrees

nuclear 7.6 - cold

nuclear 8.3 - 33 degrees

100-300 ug of RNA from each fraction was centrifuged for 4-5 hours in the presence of 0.02 M NaCl. Alternate fractions of 4 and 14 drops were collected. The 4 drop fractions were diluted with water and the absorbancy at 260 mμ was measured, followed by radioactivity counting. The RNA from the 14 drop fractions was precipitated by the addition of ethanol. This was used for template activity analysis.

The sedimentation values are approximate and were estimated by comparison with known values for the ribosomal components. Total RNA (———) was computed from absorbancy measurements at 260 mμ. Template activity (- - -) was expressed as the increase in μμ moles of leucine incorporated per fraction of RNA. Pulse labeling (—x———x) was expressed as the number of counts per minute per mg RNA in each fraction.

DISCUSSION

The regenerative process in the liver offers an experimental situation wherein the various physiologic features of the cell are called upon to function under a stress. The normal liver cell concerns itself mainly with the maintenance of homeostasis in the intact organ, synthesizing nuclear and cytoplasmic proteins as needed. In sharp contrast, the liver cell in a compromised organ - one in which 70% of the parenchyma has been removed - must concern itself with the reparative processes of constructing new cells and their contents in addition to maintaining a dynamic equilibrium. Thus, it was hoped that the study of messenger RNA would be facilitated by examining regenerating liver and comparing the findings to those established for normal liver.

Early experiments concerned the definition of conditions best suited to such an undertaking. Several interesting relationships were discovered during this initial process. One such finding was the discovery that upon heating the phenol reaction mixture, the extraction of high pH RNA relatively free of contamination from DNA was possible. Also, this finding permitted the appreciation of the fact that high pH RNA was extremely rich in template activity. Furthermore, with the application of these techniques to both nuclear and cytoplasmic fractions independently, together with the pulse labeling of the RNA, information was obtained which allowed one to speculate upon the cellular localization of the RNA with messenger activity.

The experiments which offered the most interesting data were those in

which the sedimentation patterns of template activity and pulse labeling were studied. In many respects there were no significant differences or merely inconstant variations between regenerating and normal liver. However, certain reproducible findings were observed only in regenerating tissue.

In the nuclear 8.3 sedimentation patterns, there is a polydispersity of the template activity which appears to be less pronounced in normal liver. This may be a function of the greater variety of proteins required in the regenerative process. Also, in nuclear 8.3 regenerating liver there is a larger amount of radioactivity in the heavy region of the gradient (28 S) as compared to normal liver where the bulk of the radioactivity is confined to the 9-16 S zone. In contrast, major peaks of template activity are localized in the lighter regions of the gradients (9-16 S). This probably indicates that regenerating liver synthesizes predominantly ribosomal RNA while normal liver synthesizes predominantly messenger RNA. Data consistent with this interpretation come from the study of base compositions of the RNA in the nuclear 8.3 fraction[56]. It was shown that the material in the heavy region of the gradient resembles ribosomal RNA while the material in the light region (9-16 S) has a DNA-like base composition for both regenerating and normal liver systems.

The feature of major interest in the neutral pH nuclear fraction was the sedimentation pattern of early-labeled RNA. This material consistently sedimented in a zone heavier than the 28 S RNA and heavier than the template

activity in the same region. This heavy radioactive peak is thought to be similar to one in the heavy region of the nuclear 8.3 fraction. This material probably represents precursor ribosomal RNA consistent with findings that ribosomal precursors are heavier than 28 S[39, 55].

The cytoplasmic 8.3 fraction was noteworthy in that it was the only fraction in regenerating liver that had a lower specific radioactivity than normal liver. This finding might be due to the increased amounts of 18 S material. If this RNA were functioning as precursor ribosomal RNA, then it would be expected to demonstrate a higher degree of labeling, especially in regenerating liver where there is increased ribosomal synthesis. However, since it has less radioactivity, this RNA probably serves some other function and is maintained at higher levels for use in the regenerative process. At the stage of regeneration when the pulse is given (23rd-24th hour), this material must not be rapidly turning over.

Finally, the absence of a shift in the material with template activity in the 38 degree cytoplasmic 7.6 fraction should be commented upon. In normal liver, template activity shifts from the 18 S to the 28 S region upon heating[51, 52, 46]. The explanation for this shift concerns the formation of a postulated ribosomal-template complex[46]. In these studies, it was noted that many factors - such as minor changes in temperature, pH, centrifugation time, buffers - caused variability in the sedimentation patterns. Therefore, whether or not this difference

in regenerating liver is due to experimental variability or whether it is due to an inherent property of regenerating tissue cannot be stated with certainty.

While this investigation did serve to isolate and characterize various species of RNA from the nucleus and cytoplasm of regenerating rat liver, the real significance of each of the RNA patterns can still only be alluded to. It is true that the RNA described in this paper with high amounts of early labeling and concomitant template activity in the 9-16 S sedimentation range does satisfy many of the criteria for that of messenger RNA. However, further experimentation is needed to elucidate the base compositions of these RNA species to allow more exacting identification. Most importantly, in order to be able to study the properties of an RNA with messenger function, it will be necessary to investigate this species from a more functional approach rather than from a purely physical approach. Whether or not a fraction of messenger RNA has significance will ultimately be determined by its characterization not only by those prerequisites set forth by Jacob and Monod[26], but also by what meaningful purpose this RNA actually serves in the complex metabolic machinations of the cell.

SUMMARY

Pulse-labeled RNA from the cytoplasm and purified nuclei were obtained from regenerating rat livers. Using a phenol extraction procedure at neutral pH (7.6) and at high pH (8.3), at 4 degrees C and at 38 degrees C, various RNA species were isolated. The material was subjected to sucrose gradient zone centrifugations and the RNA fractions were analyzed for both template activity and radioactivity. The sedimentation patterns were compared to those established for normal liver.

1. Heating the phenol extraction mixture to 38 degrees C allows extraction of high pH RNA free of contamination by DNA. This permitted an accurate evaluation of template activity in high pH nuclear and cytoplasmic fractions.

2. The bulk of the RNA is extracted from the cytoplasm at neutral pH (7.6). There is more cytoplasmic 7.6 RNA in regenerating liver. The highest specific radioactivity is found in the nuclear 8.3 fraction. This is consistent with results in normal liver.

3. In the nuclear 8.3 sedimentation patterns, there is a greater degree of polydispersity of template activity in regenerating liver than in normal liver. This was thought to be a function of the greater variety of proteins needed for the regenerative process.

4. The bulk of the radioactivity in the nuclear 8.3 fraction sediments in the 28 S region. In normal liver, it sediments in the 9-16 S zone. In both experimental systems, the major portion of template activity resides in the 9-16 S region.

This was interpreted as indicating that regenerating liver synthesizes predominantly ribosomal RNA and normal liver, predominantly messenger RNA.

5. A major peak of RNA with high radioactivity was found to sediment in a zone heavier than 28 S in the regenerating nuclear 7.6 fraction. This material probably represents precursor ribosomal RNA.

6. The cytoplasmic 8.3 fraction is the only fraction in regenerating liver with a lower specific radioactivity than its equivalent in normal liver. This suggested that this material was not functioning as precursor ribosomal RNA, but served another function in regeneration.

7. No shift in template activity to a heavier sedimentation value as reported for normal liver is noted in the heated cytoplasmic 7.6 fraction for regenerating liver. This may be due to an inherent property of regenerating liver or may be due to experimental variability.

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